P1 1127669

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March 01, 2004

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APPLICATION NUMBER: ***60/432,219***
FILING DATE: ***December 09, 2002***
RELATED PCT APPLICATION NUMBER: *PCT/US03/39067*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

W. Montgomery

W. MONTGOMERY
Certifying Officer

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
George R.	Pettit	Paradise Valley, Arizona

☒ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Narcistatin Prodrugs

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 21
☐ Drawing(s) *Number of Sheets*
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.
☒ A check or money order is enclosed to cover the filing fees
☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 060590 Fennemore Craig
☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): 160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☒ Yes, the name of the U S Government agency and the Government contract number are: CA-44344-03-12 and CA-90441-01

Respectfully submitted,

SIGNATURE [signature]
TYPED or PRINTED NAME Susan Stone Rosenfield
TELEPHONE (602) 916-5317

Date 12/9/02

REGISTRATION NO. *(if appropriate)* 36,287
Docket Number: 12504.391

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	12504.391	Type a plus sign (+) inside this box →	+

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Noeleen	Melody	Mesa, Arizona

Number 2 of 2

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FENNEMORE CRAIG

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December 9, 2002

VIA EXPRESS MAIL (EV 130103488 US)

Box Provisional Patent Application
Commissioner for Patents
Washington, D. C. 20231

Re: Submission of a New United States Provisional Patent Application
Title: NARCISTATIN PRODRUGS
Inventor: Pettit, et al.
Our File No.: 12504.391

Dear Sir:

We hereby submit the following documents concerning the referenced patent application:

1. Fee Transmittal Form for FY 2002 (PTO/SB/17);
2. Provisional Application for Patent Cover Sheet (PTO/SB/16);
3. Provisional Patent Application, including specification (21 pages);
4. Assignment and Cover Sheet for Recording;
5. Check No. 235037 for $ 160.00 to cover the Application filing fee;
6. Check No. 235654 for $40.00 to cover the Assignment recording fee;
7. Power of Attorney; and
8. Postage-paid postcard acknowledging receipt of this letter and the foregoing.

FENNEMORE CRAIG

Please accord this application a serial number and a filing date. The Commissioner is hereby authorized to charge any additional fee required or credit any overpayments to Deposit Account No. 060590.

Respectfully submitted,

By _____
Susan Stone Rosenfield
FENNEMORE CRAIG
Registration No. 36,287

Express Mail Label No. EV 130103488 US Date of Deposit 12/9/2002

I hereby certify that this paper and all documents and any fee referred to herein are being deposited on the date indicated above with the U.S. Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10, and is addressed to Box Provisional Patent Application, Commissioner for Patents, Washington, D.C. 20231.

_____ 12/9/2002
Louis A. Lofredo, Legal Assistant Date of Signature

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FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision

TOTAL AMOUNT OF PAYMENT	($) 200

Complete If Known	
Application Number	Unassigned
Filing Date	November 20, 2002
First Named Inventor	Pettit, et al.
Examiner Name	Unassigned
Group Art Unit	Unassigned
Attorney Docket No.	12504.391

METHOD OF PAYMENT

1. [X] The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:
   - Deposit Account Number: 060590
   - Deposit Account Name: Fennemore Craig
   - [X] Charge Any Additional Fee Required Under 37 CFR 1 16 and 1 17
   - [X] Applicant claims small entity status See 37 CFR 1 27
2. [X] **Payment Enclosed:** [X] Check [ ] Credit card [ ] Money Order [ ] Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	160

SUBTOTAL (1) ($) 160

2. EXTRA CLAIM FEES

	Extra Claims		Fee from below	Fee Paid
Total Claims	0	-20** = 0	X 0	= 0
Independent Claims	0	- 3** = 0	X 0	= 0
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

**or number previously paid, if greater, For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	40.00
146	740	246	370	Filing a submission after final rejection (37 CFR § 1 129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1 129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 40

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	Susan Stone Rosenfield	Registration No. (Attorney/Agent)	36,287	Telephone	602 916-5317
Signature				Date	12-9-02

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United States Provisional Patent Application

Title: Narcistatin Prodrugs

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Express Mail Label No. EV 130103488 US Date of Deposit 12/9/02

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Louis A Loreda, Legal Assistant

12/9/02
Date of Signature

SROSENFI/1362411.2/12504 391

INTRODUCTION

Financial assistance for this invention was provided by the United States Government, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Department of Health and Human Services, Outstanding Investigator Grant CA44344-03-12 and CA90441-01; the Arizona Disease Control Research Commission; and private contributions. Thus, the United States Government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to a novel compounds, and methods for synthesizing same, which show promising utility in the treatment of cancer. The compound described herein has been denominated narcistatin. Further described herein are numerous derivatives of narcistatin.

BACKGROUND OF THE INVENTION

Over 30 species representing 11 genera (among 85 total) of the plant family Amaryllidaceae have been employed in traditional treatments for human cancer. Such applications of certain *Narcissus* species were recorded as early as 200 B.C. Pettit, G. R. *et al., J. Nat. Prod.* **1995,** *58,* 756-759; Pettit, G. R., *et al., J. Nat. Prod.,* **1995,** *58,* 37-43. The biologically active constituents of Amaryllidaceae species have been under investigation from at least 1877 following Gerrard's report on a component of *Narcissus pseudonarcissus* designated narcissia. Gerrard, A. W., *Pharm. J.,* **1877,** *8,* 214; Cook, J. W., In *The Alkaloids*, Manske, R. H. F.; Holmes, H. L., Ed.; Academic Press: New York, 1952; pp. 331. Presently, some 48 alkaloids and carbostyrils bearing a variety of carbon skeletons have been isolated from Narcissus species. Weniger, B., *et al., Planta Med.,* **1995,** *61*, 77-79. Of these, the isocarbostyrils narciclasine **(1)** and pancratistatin **(2)** have been found to display the most promising *in vivo* antineoplastic activities and a selection of other amaryllidaceae alkaloids have been shown to provide cancer

cell growth inhibitory activity. Pettit, G. R., *et al., J. Nat. Prod.*, **1995**, *58*, 756-759; Pettit, G. R., *et al., J. Nat. Prod.*, **1995**, *58*, 37-43; Pettit, G. R.., *et al., J. Org. Chem.*, **2001**, *66*, 2583-2587; Rigby, J. H., *et al., J. Amer. Chem. Soc.*, **2000**, *122*, 6624-6628; Suffness, M., *et al.*, In The Alkaloids, Drossi, A., Ed., Academic Press: New York, 1985; pp. 205-207; Youssef, D. T. A., *et al., Pharmazie* **2001**, *56*, 818-822.

Pancratistatin (**2**), which we first discovered in *Pancratium littorale* (reidentified as *Hymenocallis littoralis*) and later in *Narcissus* species, has been undergoing extended preclinical development. Pettit, G. R., *et al., J. Org. Chem.*, 2001, 66, 2583-2587; Rigby, J. H., *et al., J. Amer. Chem. Soc.* 2000, 122, 6624-6628; Pettit, G. R., *et al., J. Nat. Prod.*, 1995, 58, 756-759; Pettit, G. R.., *et al., J. Nat. Prod.*, 1995, 58, 37-43. That very important initiative was greatly assisted by conversion of the sparingly soluble isocarbostyril to a 7-O-phosphate salt. Pettit, G. R., *et al., Anti-Cancer Drug Design* 2000, 15, 389-395; Pettit, G. R., *et al., Anti-Cancer Drug Design* 1995, 10, 243-250. The antimitotic activity of narciclasine (**1**) has been known for over 35 years. Subsequently, it was shown in U.S. National Cancer Institute research to be active against *in vivo* growth of the M5076 sarcoma and P388 lymphocytic leukemia. In addition, it was found to inhibit protein synthesis in Erlich asciter cancer cells. Suffness, M., *et al.*, The Alkaloids, Drossi, A., Ed., Academic Press: New York, 1985; pp. 205-207. However, as with the closely related pancratistatin (**2**) the low solubility properties of narciclasine has contributed to the delay in its preclinical development. Most of our early investigation involving this potentially useful isocarbostyril have targeted its use as a starting point for a practical synthesis of pancratistatin (**2**) and for SAR purposes. Pettit, G. R., *et al., J. Org. Chem.* 2001, 66, 2583-2587; Rigby, J. H., *et al., Amer. Chem. Soc.* 2000, 122, 6624-6628; Pettit, G. R., *et al., J-C. Heterocycles* 2002, 56, 139-155. Now we are pleased to report a very convenient transformation of narciclasine (**1**) to water soluble cyclic phosphate prodrugs (**3**).

SUMMARY OF THE INVENTION

An efficient procedure was found for synthetic conversion of the sparingly soluble anticancer isocarbostyril narciclasine (**1**), a component of various *Narcissus* species, to a cyclic-phosphate designated narcistatin (**3b**). The reaction between narciclasine, tetrabutylammonium dihydrogen phosphate, dicyclohexylcarbodiimide, and *p*-toluenesulfonic acid in pyridine afforded pyridinium narcistatin (**3a**) in reasonable yields. Preparation of sodium narcistatin (**3d**) was achieved by two methods. Procedure A involved the transformation of narcistatin (**3a**) into the water soluble prodrug (**3d**) and other salt derivatives by cation exchange chromatography. Procedure B allowed sodium narcistatin (**3d**) to be obtained in high yield, following cation exchange chromatography, from the reaction between narciclasine, tetrabutylammonium dihydrogen phosphate and dicyclohexylcarbodiimide in pyridine.

Narcistatin (**3b**) and fifteen salt derivatives were evaluated against a panel of human cancer cell lines and the range (0.1 - 0.01) of GI_{50} values in μg/ml was found to parallel that shown by the parent narciclasine. In summary, the very successful conversion of narciclasine to a water soluble (60 mg/ml for sodium salt **3d**) cyclic phosphate prodrug will now allow this potentially useful *Narcissus* anticancer component to be further developed.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the x-ray structure of pyridinium narcistatin (3a).

Figure 2 illustrates the x-ray cell contents of pyridinium narcistatin hydrate (3a).

DETAILED DESCRIPTION OF THE INVENTION

Early experience by one of the inventors in nucleotide chemistry involving phosphate esters and cellular phosphatases combined with recent successes in synthesis of phosphate prodrugs made such an approach most attractive for obtaining a water soluble narciclasine prodrug. Pettit, G. R. Synthetic Nucleotides, Van Nostrand Reinhold Co: New York, 1972; Pettit, G. R., *et al.,* Anti-Cancer Drug Design 2000, 15, 389-395; Pettit, G. R., *et al.,* Anti-Cancer Drug Design 1995, 10, 243-250; Pettit, G. R., *et al.,* Anti-Cancer Drug Design 2000, 15, 397-403; Saulnier, M. G., *et al.,* Med. Chem. Lett. 1994, 4, 2567-2572; Ueda, Y., *et al.,* Med. Chem. Lett. 1995, 5, 247-252. However, a selection of the more obvious methods such as $POCl_3$, or 2-cyanoethylphosphate with dicyclohexylcarbodiimide (DCCI), and various unprotected or protection (e.g. narciclasine 3,4-acetonide) strategies involving narciclasine (**1**) only led to unpromising mixtures. Pettit, G. R., *et al.,* Anti-Cancer Drug Design 2000, 15, 389-395; Pettit, G. R., *et al.,* Anti-Cancer Drug Design 1995, 10, 243-250; Taktakishvili, M., *et al.,* Tetrahedron Lett. 2000, 41, 7173-7176; Tener, G. M., J. Amer. Chem. Soc. 1961, 83, 159-168; Scheit, K. H., Nucleotide Analogs, Synthesis and Biological Function; Wiley-Interscience: New York, 1972; Khorana, H. G., *et al.,* J. Chem. Soc. 1953, 2257-2260; Khorana, H. G. J. Amer. Chem. Soc. 1954, 76, 3517-3527; Dekker, C. A., *et al.,* J. Amer. Chem. Soc. 1954, 76, 3522-3527; Tener, G. M.; Khorana, H. G., J. Amer. Chem. Soc. 1955, 77, 5348. Eventually, we examined use of the readily soluble tetrabutylammonium dihydrogen phosphate in pyridine as the phosphate source. Initially, the phosphate failed to couple with narciclasine in the presence of DCCI until three equivalents of *p*-toluenesulfonic acid was employed to promote condensation, at which point precipitation of dicyclohexylurea (DCU) began. When the reaction mixture was heated to 80°C, the pyridinium salt of narciclasine-3,4-cyclic phosphate **3a** (herein designated pyridinium

narcistatin), precipitated. Following collection of precipitated DCU and the narcistatin pyridinium salt, the solids were titrated with water to dissolve the cyclic phosphate (**3a**). Concentration of the water fraction afforded the pyridinium salt in 40% yield. The mother liquor was concentrated to a brown oil and added to a large volume of water; an immediate precipitate was observed. The solution was filtered and the filtrate was found to be primarily unreacted narciclasine with some DCU as impurity. The reaction did not go to completion even after prolonged stirring and addition of more reagents.

Examination of the ^{1}H-NMR (DMSO-d_6) spectrum of the pyridinium salt **3a** showed a multiplet corresponding to the signals for four protons at 4.42-4.31 ppm and a doublet of doublets corresponding to the signal for one proton at 4.15 ppm. Assuming four ring hydrogens resonating in this region, the signal for H-1 was assigned downfield at 6.5 ppm. Only one of the signals corresponded to a hydroxyl group. A D_2O experiment resulted in a considerable change in the splitting pattern of the multiplet at 4.3 ppm and 8.60 ppm, suggesting loss of the OH signal and NH-5 signal, respectively. Other signals at 13.66 and 9.00 were also absent from the D_2O treated spectrum due to deuterium exchange with OH-7 and pyridinium NH. The ^{31}P-NMR (DMSO-d_6) spectrum gave one signal at 20.3 ppm suggesting only one phosphorus atom, this together with the ^{1}H NMR data suggested the formation of the cyclic phosphate. However, despite extensive 2D NMR experiments, the position of the phosphate could not be established unambiguously. Consequently, narciclasine pyridinium salt (**3a**) was recrystallized from pyridine-water and examined by X-ray crystallography to establish the 3,4-cyclic phosphate structure. The resulting structure of **3a** is depicted in Figure 1. In addition to two pyridinium cations and two cyclic phosphate anions, the unit cell was found to contain three molecules of water solvate, as shown in Figure 2.

In order to extend the narcistatin cation series, phosphoric acid **3b** was prepared by dissolving the pyridinium narcistatin in water and passing it through a column containing Dowex 50W X8 200 cation exchange resin (hydrogen form). A solution of the pyridinium narcistatin in water was also used to prepare the lithium (**3c**), sodium (**3d**) (procedure A), potassium (**3e**) and cesium (**3g**) salts of narcistatin by passage through a Dowex 50W X2 column bearing the respective cations. The magnesium (**3g**), calcium (**3h**), zinc (**3i**), and manganese (**3j**) salts were obtained by suspending phosphoric acid **3b** in methanol-water (3:2) and adding 0.5 equivalent of the respective metal acetate in water. The resulting opaque solution was stirred for several days as the salt precipitated from solution. These dication salts proved to be only sparingly soluble in water. A selection of ammonium salts were prepared by allowing phosphoric acid **3b** to react with the respective amine (1.2 equiv) at room temperature. The reaction mixture was concentrated and product precipitated to give ammonium salts **3k-o**. Procedure B for the preparation of sodium narcistatin **3d** is as follows. The reaction between narciclasine, tetrabutylammonium dihydrogen phosphate and DCCI in pyridine was carried out at 80°C without the addition of the para-toluene sulfonic acid. The reaction was monitored by ^{1}H NMR and found to go to completion in four days with addition of more reagents at 24 hours. Isolation followed by cation exchange chromatography gave sodium narcistatin in high yield (88%).

Narciclasine cyclic phosphate prodrugs **3a-o** were evaluated against a minipanel of human cancer cell lines and the murine P388 lymphocytic leukemia. Results of the cancer cell line evaluation of narcistatins **3a-o** appears in Table 1. The GI_{50} 0.1-0.02 μg/ml strong activity range parallels that already reported for the parent, narciclasine (**1**). Pettit, G. R.; Melody, N.; Herald, D. L. J. Org. Chem. 2001, 66, 2583-2587

Experimental Section.

Narciclasine (1) was isolated form *Hymenocallis littoralis* (Jacq.) Salisb, (Amaryllidaceae) grown by our group in Tempe, Arizona. Pettit, G. R., *et al.*, J. Nat. Prod. 1995, 58, 756-759; Pettit, G. R., *et al.*, J. Nat. Prod. 1995, 58, 37-43. Reagents were purchased from Aldrich Chemical unless otherwise noted and used as received. Solvents were distilled prior to use and pyridine preceding distillation was dried over potassium hydroxide pellets. Dowex 50X8-200 and Dowex 50WX2 cation exchange resins (H^+ form) were washed with methanol, 1 *N* hydrochloric acid and deionized water. The cation forms of the resin were obtained by washing with a 1 *N* solution of the appropriate base followed by deionized water. DEAE SEPHADEX A-25 weak anion exchange resin (acetate form) was purchased from the Sigma-Aldrich Company and was washed with 1 *N* triethylammonium bicarbonate (TEAB) solution and then equilibrated with 10 m*N* TEAB buffer solution.

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Thin layer chromatography was performed on Analtech silica gel GHLF plates, the narciclasine containing derivatives were visible as green-blue fluorescent spots under long wave ultraviolet light, and were rendered permanent by staining with iodine vapor. Phosphorous containing compounds were detected using the modified Jungnickel's reagent (perchloric acid - malachite green - sodium molybdate) developed by Vaskovsky and Latshev. Khorana, H. G., *et al.*, A. R. J. Chem. Soc. 1953, 2257-2260; Khorana, H. G., J. Amer. Chem. Soc. 1954, 76, 3517-3527; Dekker, C. A., *et al.*, H. G. J. Amer. Chem. Soc. 1954, 76, 3522-3527; Tener, G. M., *et al.*, J. Amer. Chem. Soc. 1955, 77, 5348. Optical rotation values were recorded using a Perkin Elmer 241 polarimeter. High resolution FAB spectra were obtained using a JEOL LCMate magnetic sector instrument in either the FAB mode, with a glycerol matrix, or by APCI with a polyethylene glycol reference. All ^1H NMR spectra were obtained using a Varian Gemini 300

MHz instrument unless otherwise noted. The ^{13}C, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HMQC, and ^{31}P-NMR experiments were conducted employing a Varian Unity 500 MHz instrument.

Pyridinium Narcistatin (3a)

Narciclasine 1 (1.0 g, 3.4 mmol) was added to pyridine (50 ml) and the solution was heated to 80°C. Next, tetrabutylammonium-dihydrogen phosphate (5.13 g, 15.11 mmol, 4.4 equiv), dicyclohexylcarbodiimide (5.0, 24.5 mmol, 7.0 equiv) and *p*-toluenesulfonic acid (3.0 g, 15.8 mmol, 4.63 equiv, added slowly) were added. After 2g of the sulfonic acid was added, a precipitate began to separate. The reaction mixture was stirred under argon at 80°C for 2.5 hours. The precipitate was collected and washed with methanol to remove pyridine. The precipitated cyclic phosphate (3a) was separated from the DCU by washing with water (200 ml). The aqueous filtrate was concentrated to an off-white solid and dried (vacuum) overnight to yield 0.59 g, 40.4%. The mother liquor was concentrated to a brown oil and water (750 ml) added. An immediate precipitate was observed, which was collected and dried to 0.75 g of white solid. The ^{1}H NMR (DMSO-d_6) showed this material to be recovered starting material with a small amount of DCU impurity. Recrystallization of phosphate 3a from pyridine-water gave crystals that were used for X-ray crystallography. $[\alpha]^{26}_{D} = -6.4°$ (c 0.44, DMSO); m.p. 275°C; ^{1}H NMR (DMSO -d_6, 500 MHz) δ 13.66 (s, 1H), 9.00 (s, 1H), 8.60 ppm (m, 3H), 7.9 (t, J = 7.5 Hz, 1H), 7.5 (m, 2H), 7.04 (s, 1H), 6.5 (s, 1H), 6.06 (d, J = 3 Hz, 2H), 4.42-4.31 (m, 4H), 4.15 (dd, J_{14}, 6.5 Hz, 1H); ^{13}C NMR (DMSO, 500 MHz) δ 167.7, 152.6, 148.6(2), 145.2, 137.4(2), 133.5, 128.5, 126.9, 125.3, 124.4, 104.3, 102.1, 94.3, 76.9, 76.7, 70.4, 53.9; ^{31}P (DMSO-d_6, 200 MHz) 20.3 (s, 1P); found by HRAPCI (negative ions) mass spec. 368.0179, calc. for $C_{14}H_{11}O_9NP$ 368.2164.

Crystal Structure of Pyridinium Narcistatin (3a).

X-Ray Crystal Structure Determination. Pyridinium narcistatin hydrate (3a): A thin plate (~0.07 x 0.35 x 0.54 mm), grown from pyridine/water solution, was mounted on the

tip of a glass fiber. Cell parameter measurements and data collection were performed at 123 K with a Bruker SMART 6000 diffractometer system using Cu Kα radiation. A sphere of reciprocal space was covered using the multirun technique. SMART for Windows NT v5.605; BrukerAXS Inc.: Madison, WI, 2000. Thus, six sets of frames of data were collected with 0.396° steps in ω, and a last set of frames with 0.396° steps in φ, such that 91.7% coverage of all unique reflections to a resolution of 0.84A was accomplished.

Crystal Data: $C_{14}H_{11}NO_9P \bullet C_5H_6N \bullet 1\ ½\ H_2O$ (hydrate), M_r=475.34, triclinic, P1, a=7.4949(1), b=8.0371(1), c=16.9589(2) A, α=85.248(1), β = 83.243(1), γ=79.383(1)°, V=994.60(2) A^3, Z=2, ρ_c=1.577 Mg/m^3, μ(CuKα) = 1.837 mm^{-1}, λ= 1.54178 A, F(000)=494.

A total of 7587 reflections was collected, of which 4733 reflections were independent reflections (R(int) = 0.0273). Subsequent statistical analysis of the data set with the XPREP program indicated the spacegroup was P1. XPREP-The automatic space group determination program in the SHELXTL. (SHELXTL-NT Version 5.10; BrukerAXS Inc., Madison, WI, 1997: an integrated suite of programs for the determination of crystal structures from diffraction data. This package includes, among others, XPREP (an automatic space group determination program), SHELXS (a structure solution program via Patterson or direct methods), and SHELXL (structure refinement software)). Final cell constants were determined from the set of the 4564 observed (>2σ(I)) reflections which were used in structure solution and refinement. An absorption correction was applied to the data with SADABS. Blessing, R. Acta Crystallogr. 1995, A51, 33-38. Structure determination and refinement was readily accomplished with the direct-methods program SHELXTL. SHELXTL-NT Version 5.10; Bruker AXS Inc.: Madison, WI, 1997. An integrated suite of programs for the determination of crystal structures from diffraction data. This package includes, among others, XPREP (an automatic space group determination program), SHELXS (a structure solution program via Patterson or direct methods),

and SHELXL (structure refinement software). All non-hydrogen atom coordinates were located in a routine run using default values for that program. The remaining H atom coordinates were calculated at optimum positions, except for water hydrogen atoms, which were located *via* difference maps. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement procedure. The H atoms were included, their Uiso thermal parameters fixed at either 1.2 or 1.5 (depending on atom type) the value of the Uiso of the atom to which they were attached and forced to ride that atom. The final standard residual R_1 value for **3a** was 0.0393 for observed data and 0.0403 for all data. The goodness-of-fit on F^2 was 1.053. The corresponding Sheldrick R values were wR_2 of 0.1074 and 0.1099, respectively. The final model used for pyridinium narcistatin **3a** is shown in Figure 1. In addition to the parent molecules (i.e., two narcistatin anions and two pyridinium cations) in the unit cell, three molecules of water solvate were also present. One of these water molecules was disordered over two sites, each of which were given 0.5 site occupancies. A final difference Fourier map showed minimal residual electron density; the largest difference peak and hole being +0.350 and -0.255 e/Å^3, respectively. Final bond distances and angles were all within expected and acceptable limits.

Narcistatin (3b).

A solution of pyridinium narcistatin (**3a**, 0.05 g) in water (2 ml) was obtained by heating (water bath) at 60°C and allowing the solution to cool prior to passing through a column prepared from Dowex 50X8-200 cation exchange resin (hydrogen form). A suspension began to form in the column as the phosphoric acid (**3b**) formed. The column was eluted with water and phosphoric acid **3b** eluted as a milky white suspension. The combined fractions containing phosphoric acid **3b** were freeze dried to afford the product as a colorless solid, (36 mg, 86%); m.p. 175°C (dec.); ^{1}H NMR (DMSO-d_6, 300 MHz), δ 13.65 (s, 1H), 9.02 (s, 1H), 7.06 (s, 1H),

6.48 (s, 1H), 6.17 (d, J_{ab} = 10.2 Hz, 1H), 6.06 (m, 2H),4.46-4.30 (m, 3H), 4.18 (m, 1H); calc for $C_{14}H_{13}NO_9P$ 370.0328; found by HR (APCI) $[M+H]^+$ 370.0361.

General Procedure for Preparation of Narcistatin Prodrugs 3c-f.

Pyridinium narcistatin (**3a**, 50 mg) was dissolved in water (35 ml) and the solution passed through a column (1 x 20 cm) of Dowex 50W-X2 bearing the respective cation. The u.v. active fractions were combined and freeze dried to give the corresponding narcistatin salt as a colorless solid unless otherwise recorded. The solubility of each in water (mg/ml) now follows: **3c**, >50 mg; **3d**, 60 mg; **3e**, 11 mg; **3f**, <13 mg.

Lithium Narcistatin (3c).

Yield, 65 mg, 77%; m.p. 220°C (dec); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.79 (s, 1H), 8.71 (s, 1H), 7.07 (s, 1H), 6.49 (s, 1H), 6.13 (m, 2H), 4.36 (m, 2H), 4.04 (m, 1H), 3.93 (m, 1H); ^{13}C NMR (DMSO-d_6, 300 MHz), 167.6, 152.5, 145.2, 133.3, 129.1, 127.3, 125.6, 104.3, 101.9, 94.2, 75.2, 74.6, 70.4, 53.8.

Sodium Narcistatin (3d). (Procedure A).

Colorless solid, 38 mg, 87%); $[\alpha]^{25}_D$ = -6.33 (c 0.3, DMSO); m.p. 275°C; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.72 (s, 1H) 8.63 (s, 1H), 6.99 (s, 1H), 6.41 (s, 1H), 6.05 (m, 2H), 5.77 (bs, 1H), 4.26 (m, 2H), 3.4 (m, 1H), 3.83 (m, 1H); ^{13}C NMR (DMSO-d_6, 500 MHz), 167.6, 152.5, 145.2, 133.3, 129.1, 127.3, 125.5, 104.3, 101.9, 94.2, 75.2, 74.5, 70.4, 53.9; ^{31}P (DMSO-d_6, 200 MHz) 16.98.

Sodium Narcistatin (3d). Procedure B.

Narciclasine 1 (0.113 g, 0.368 mmol) was added to pyridine (4ml) and the solution heated to 80°C. Next, tetrabutylammonium dihydrogen phosphate (0.075 g, 0.22 mmol, 0.6 equiv.) and dicyclohexylcarbodiimide (0.4 g, 1.93 mmol, 5 equiv.) were added. The reaction mixture was stirred under argon at 80°C for 24 hours. Tetrabutylammonium dihydrogen

phosphate (0.185 g) was added followed by DCCI (0.4 g) and the reaction stirred for a further 72 hours. ^{1}HNMR (DMSO-d_6) of the crude reaction mixture showed complete conversion to product. The reaction was cooled and filtered. Water (100 ml) was added to the mother liquor, which was then filtered to remove any precipitated DCU. The aqueous solution was then concentrated to minimum volume. The solution was then eluted on an ion exchange column of Dowex 50WX8-200 (sodium form) and the UV active fractions were combined and freeze dreied to afford the product as a white solid (0.113 mg, 88%). Comparison of the ^{1}HNMR of this product in DMSO-d_6 with the narcistatin sodium salt **3d** prepared from the pyridinium narcistatin **3a** by the method outlined above showed them to be identical. This method is more practical and dramatically improves the yield of narcistatin from narciclasine.

Potassium Narcistatin (3e)

Off-white solid, 59 mg, 80%, m.p. 250°C, ^{1}H NMR (DMSO-d_6, 300 MHz) δ 13.74 (s, 1H), 8.65 (s, 1H), 6.98 (s, 1H), 6.40 (s, 1H), 6.04 (d, J_{ab} = 2.4 Hz, 2H), 5.74 (bs, 1H), 4.25 (m, 2H), 3.9 (m, 1H), 3.78 (m, 1H).

Cesium Narcistatin (3f)

Off white solid, 51 mg, 91%, m.p. 245°C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 13.74 (s, 1H), 8.65 (s, 1H), 6.98 (s, 1H), 6.40 (s, 1H), 6.04 (m, 2H), 5.74 (bs, 1H), 4.25 (m, 2H), 3.92 (m, 1H), 3.79 (m, 1H).

An alternative method was also developed to isolate yield narcistatin sodium salt **3d**. Narciclasine, tetrabutylammonium dihydrogen phosphate, DCCI and pyridinium *p*-toluene sulfonate were allowed to react at room temperature for 2 days. The reaction was monitored by t.l.c. using the solvent system 4:3:2:1 butanol-methanol-water-concentrated aqueous ammonia. Two major fluorescent spots were evident, narciclasine at R_f 0.65 and product at a higher R_f 0.69. Even after 4 days of stirring, the reaction was incomplete. The reaction mixture was added

to water, the DCU collected, the mother liquor was evaporated to half its volume, and 2N aqueous ammonia was added at regular intervals to maintain a pH of 8-9. The solution was passed through a column (15 x 15 cm) of Dowex 50 (pyridinium form) in order to remove the unreacted narciclasine. Narciclasine remained bound to the resin while the charged phosphate passed through unchanged. The column was then washed with methanol and the unreacted narciclasine was recovered. The cyclic phosphate was separated from contaminating inorganic phosphate by anion exchange chromatography using DEAE-Sephadex and gradient elution with aqueous triethyl ammonium bicarbonate. The triethyl ammonium salt was converted to the sodium salt by passage through a Dowex 50 column (Na^+ form). A ^{31}P-NMR confirmed the presence of a phosphate group. The yield from this reaction was 43%. Comparison of the ^1H NMR of this product in D_2O with the narcistatin sodium salt **3d** prepared from the pyridinium narcistatin **3a** by the method outlined above showed them to be identical. However, this method proved less practical and did not significantly improve the yield.

General Procedure for Preparation of Narcistatin Divalent Cation Salts 3g-j.

The experiment leading to magnesium salt **3g** provides the general method and relative quantities of reactants and solvents. In each case, the respective metal acetate was employed.

Magnesium Narcistatin (3g)

To a mixture of phosphoric acid (**3b**, 50 mg, 0.135 mmol) and methanol-water (3:2) was added a solution of magnesium acetate (15 mg, 0.0675 mmol. 0.5 equiv) in water (1 ml). The mixture became opaque immediately upon addition of the metal acetate and was stirred for 3 days while further precipitation occurred. The solution was concentrated to a white residue and water-methanol was added (1.4 ml). The precipitate was collected and dried; grey solid, m.p. 210°C dec. very insoluble in water, soluble in DMSO; ^1H-NMR (DMSO-d_6, 300 MHz) δ 13.69 (s, 1H), 8.73 (s, 1H), 6.99 (s, 1H), 6.43 (s, 1H), 6.14 (m, 1H), 6.05 (s, 2H), 5.82 (bs, 1H), 4.41 -

4.31 (m, 2H), 4.03 - 3.95 (m, 2H). Each of the divalent cation salts proved to be only sparingly soluble in water.

Calcium Narcistatin (3h)

Grey solid; 30 mg, m.p. 195°C (dec). ^{1}H NMR (DMSO-d_6, 300 MHz), δ 13.68 (s, 1H), 8.69 (s, 1H), 7.0 (s, 1H), 6.43 (s, 1H), 6.14 (d, J = 12.9 Hz, 1H), 6.05 (m, 2H), 4.29 (m, 2H), 4.02 (m, 1H), 3.94 (m, 1H).

Zinc Narcistatin (3i)

Yield of grey solid, 23 mg, m.p. 200°C (dec). ^{1}H NMR (DMSO-d_6, 300 MHz), δ 13.64 (s, 1H), 8.81 (s, 1H), 6.92 (s, 1H), 6.38 (s, 1H), 6.16 (m, 1H), 6.03 (s, 2H), 5.94 (bs, 1H), 4.31 (m, 2H), 4.20-4.17 (m, 1H), 4.07 (m, 1H).

Manganese Narcistatin (3j)

For this experiment, 41 mg of narcistatin (**3b**) was treated with manganese acetate (16 mg, 0.065 mmol. 0.5 equiv) in water (1 ml) to afford 35 mg of grey solid, m.p. 165°C (dec); ^{1}H NMR (DMSO-d_6, 300 MHz). The salt, while quite soluble in DMSO-d_6, did not give a useful spectrum.

General procedure for obtaining ammonium salts 3k-o.

Phosphoric acid **3b** (0.25 g) was dissolved in methanol-dichloromethane-water (3:1:1) (10 ml). A 2 ml aliquot of the phosphoric acid solution was added to each of the five flasks containing 1.2 equivalents of the respective amine and the reaction mixture stirred for 24 hr at rt. A precipitate separated from the reaction mixture with the quinine and imidazole examples. The solvent was concentrated and the residues reprecipitated from water-methanol to yield each of the ammonium salts **3k-o**).

Quinidinium Narcistatin (3k).

Cream-colored solid; 34 mg, m.p. 205°C (dec, 220°C melts); ^{1}H NMR (DMSO-d_6, 300 MHz), δ 13.71 (s, 1H), 8.68 (bs, 2H), 7.90 (d, J = 8.4 Hz, 1H), 7.52 (s, 1H), 7.37 - 7.40 (m, 3H), 6.99 (s, 1H), 6.4 (s, 1H), 6.13 - 6.01 (m, 3H), 5.10 (m, 4H), 4.25 (m, 2H), 3.92 (m, 5H), 3.6 - 3.2 (m, 6H), 2.42 (m, 1H), 2.2 - 2.12 (m, 1H), 1.91 - 1.84 (m, 1H), 1.60 (m, 2H), 1.47 - 1.38 (m, 1H).

Quininium Narcistatin (3l).

Cream-colored solid; 55 mg, m.p. 195°C; ^{1}H NMR (DMSO-d_6, 300 MHz), δ 13.72 (s, 1H), 8.70 (bs, 2H), 7.93 (d, J = 8.4 Hz, 1H), 7.57 (bs, 1H), 7.45 - 7.39 (m, 3H), 6.99 (s, 1H), 6.41 (s, 1H), 6.05 (m, 3H), 5.80 - 5.73 (m, 2H), 5.07 - 4.93 (m, 2H), 4.25 (bs, 2H), 4.03 - 3.85 (m, 5H), 3.38 (m, 6H), 1.91 (m, 4H), 1.71 (m, 1H), 1.47 (m, 1H).

Imidazolium Narcistatin (3m).

Off-white solid, 39 mg, m.p. 210°C; ^{1}H NMR (DMSO-d_6 300 MHz), δ 13.73 (s, 1H), 13.4 (s, 1H), 8.71 (s, 1H), 8.06 (bs, 1H), 7.21 (bm, 2H), 6.98 (s, 1H), 6.41 (s, 1H), 6.11 (bs, 1H), 6.04 (m, 2H), 4.25 (m, 2H), 3.99 (m, 1H), 3.84 (m, 1H).

Morpholinium Narcistatin (3n).

Off-white solid, 20 mg, m.p. 230°C; ^{1}H NMR (DMSO-d_6 300 MHz), δ 13.73 (s, 1H), 8.68 (s, 1H), 6.99 (s, 1H), 6.41 (s, 1H), 6.04 (d, J = 2.7 Hz, 2H), 5.76 (bs, 1H), 4.25 (bm, 2H), 3.97 (m, 1H), 3.92 - 3.71 (m, 5H), 3.03 (m, 4H), 1.22 (s, 1H).

Piperazinium Narcistatin (3o).

Off-white solid, 21 mg, m.p. 270°C; ^{1}H NMR (DMSO-d_6 300 MHz), δ 13.74 (s, 1H), 8.66 (s, 1H), 6.98 (s, 1H), 6.40 (s, 1H), 6.04 (d, J = 1.8 Hz, 2H), 5.74 (bs, 1H), 4.24 (bm, 2H), 3.93 (m, 1H), 3.81 (m, 1H), 3.14 (s, 2H), 2.83 (s, 9H).

Table 1. Solubilities, Human Cancer Cell Line and Murine P-388 Lymphocytic Inhibitory Activities of Cyclic Phosphates 3-16.

		ED_{50} (μg/ml)		GI_{50} (μg/ml)				
Compound	Solubilities[a] (mg/ml)	Leukemia P388	Pancreas-a BXPC-3	Breast MCF-7	CNS SF 268	Lung-NSC NCI-H460	Colon KM20L2	Prostate DU-145
3a	7	1.91×10^{-1}	2.2×10^{-1}	2.7×10^{-1}	1.5×10^{-1}	2.7×10^{-1}	3.4×10^{-1}	1.7×10^{-1}
3b	4	2.75×10^{-1}	3.3×10^{-1}	3.5×10^{-1}	2.2×10^{-1}	4.7×10^{-1}	5.3×10^{-1}	1.6×10^{-1}
3c	>50	1.21×10^{-1}	2.5×10^{-1}	3.1×10^{-1}	1.7×10^{-1}	3.0×10^{-1}	2.6×10^{-1}	1.3×10^{-1}
3d	60	2.55×10^{-1}	3.2×10^{-1}	5.6×10^{-1}	2.3×10^{-1}	>1	4.5×10^{-1}	1.2×10^{-1}
3e	11	2.42×10^{-1}	3.6×10^{-1}	4.0×10^{-1}	1.9×10^{-1}	6.7×10^{-1}	5.6×10^{-1}	2.6×10^{-1}
3f	<13	1.83×10^{-1}	4.1×10^{-1}	6.2×10^{-1}	3.3×10^{-1}	>1	6.6×10^{-1}	1.3×10^{-1}
3g	<1.5	1.70×10^{-1}	1.9×10^{-1}	2.5×10^{-1}	1.4×10^{-1}	2.9×10^{-1}	3.1×10^{-1}	1.0×10^{-1}
3h	<1	2.23×10^{-2}	4.5×10^{-2}	5.9×10^{-2}	3.1×10^{-2}	1.2×10^{-1}	5.9×10^{-2}	9.3×10^{-3}
3i	1.7	2.87×10^{-2}	6.9×10^{-2}	1.4×10^{-1}	5.3×10^{-2}	2.1×10^{-1}	1.6×10^{-1}	1.6×10^{-2}
3j	<3	4.27×10^{-2}	4.9×10^{-2}	7.0×10^{-2}	4.0×10^{-2}	1.5×10^{-1}	1.3×10^{-1}	3.4×10^{-2}
3k	<1	2.71×10^{-1}	3.1×10^{-1}	5.0×10^{-1}	2.5×10^{-1}	7.7×10^{-1}	5.8×10^{-1}	2.2×10^{-1}
3l	<1	3.42×10^{-2}	5.1×10^{-2}	1.2×10^{-1}	4.5×10^{-2}	1.7×10^{-1}	1.2×10^{-1}	1.3×10^{-2}
3m	5.8	2.40×10^{-1}	4.5×10^{-1}	9.0×10^{-1}	3.8×10^{-1}	>1	>1	4.4×10^{-1}
3n	>13	2.32×10^{-1}	2.5×10^{-1}	4.8×10^{-1}	2.4×10^{-1}	>1	5.4×10^{-1}	1.4×10^{-1}
3o	1.9	3.78×10^{-2}	1.0×10^{-1}	1.7×10^{-1}	9.9×10^{-2}	2.4×10^{-1}	2.2×10^{-1}	3.2×10^{-2}

[a]Solubility values were obtained using 1 ml distilled water at 25°C.

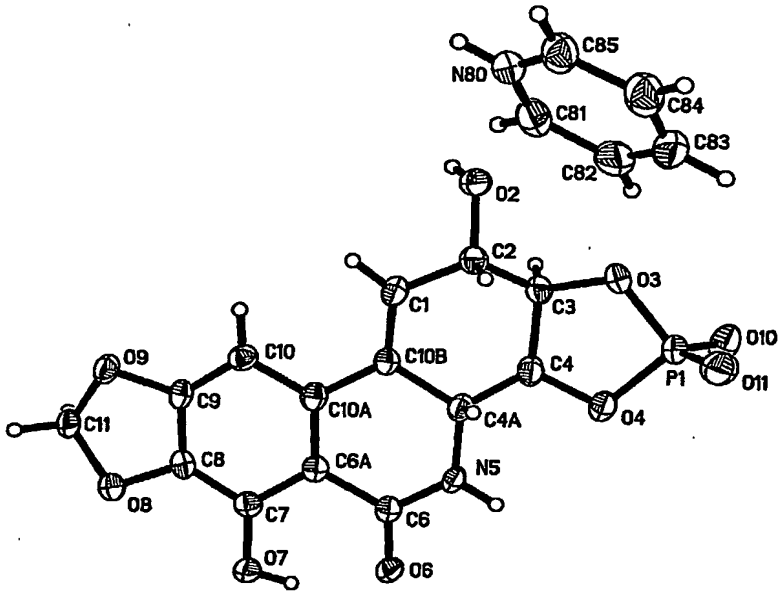

Figure 1. X-ray structure of pyridinium narcistatin (3a).

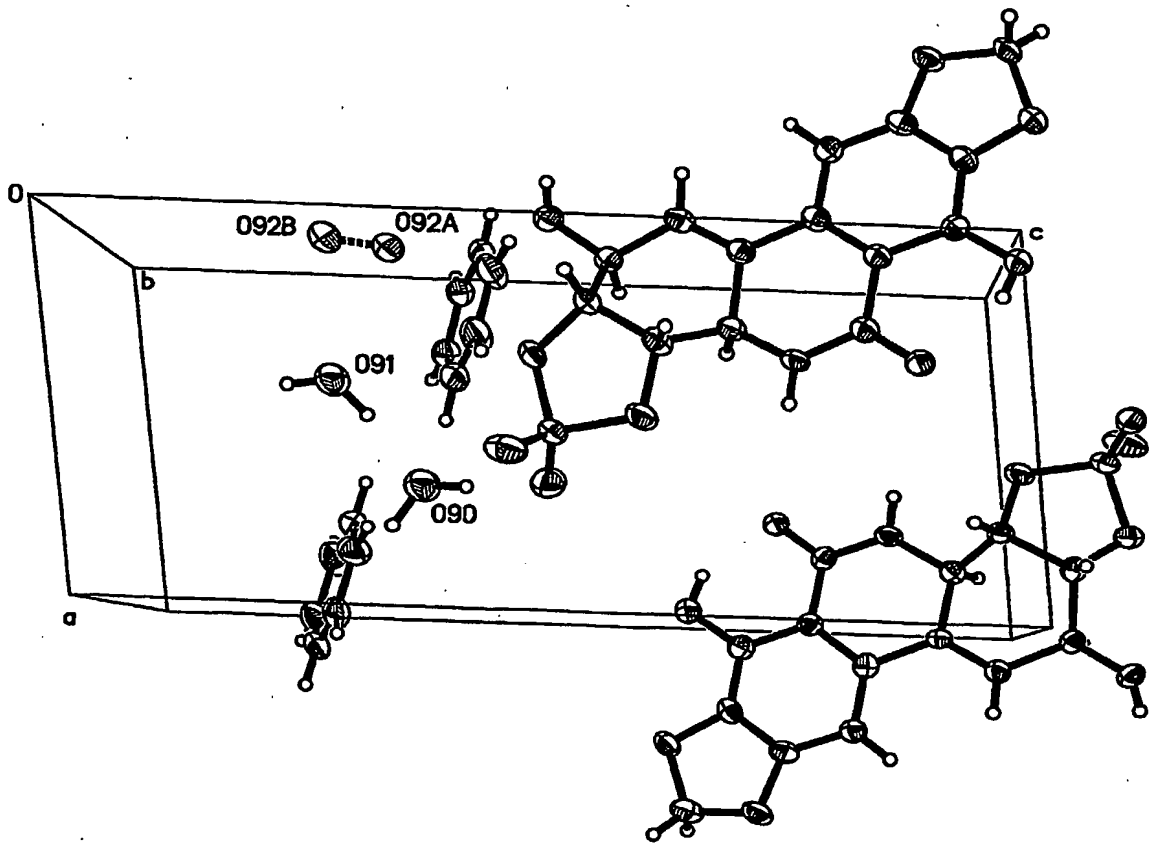

Figure 2. X-ray cell contents of pyridinium narcistatin hydrate (3a).

What we claim is:

1. The narcistatin compounds as described above.

2. A method for treating neoplastic disease comprising administering to a human subject one or more of the compounds as described above.

ABSTRACT OF THE INVENTION

The present invention provides prodrugs derived from narciclasine and having potential for use against human cancer. More specifically, disclosed is an efficient procedure for the synthetic conversion of the sparingly soluble anticancer isocarbostyril narciclasine, a component of various *Narcissus* species, to a cyclic phosphate designated "narcistatin."

Attorney's Docket No. 12504.391

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U.S. Provisional Application

Title: NARCISTATIN PRODRUGS

Inventor: Pettit, et al.

Serial No.:

Filing Date: December ___, 2002

Conf. No.:

Group Art Unit:

Examiner:

Commissioner for Patents
Washington, D.C. 20231

POWER OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS

The ARIZONA BOARD OF REGENTS, a body corporate, acting for and on behalf of ARIZONA STATE UNIVERSITY, is the Assignee of the above-captioned application pursuant to an Assignment executed on November 22, 2002, a copy of which is attached hereto. The Assignment has been or will be submitted to the Assignment Branch for recordation shortly.

The undersigned, M. Ann Freudendahl, is the Interim Director of Licensing & Intellectual Property Administration for Arizona State University, and is authorized to sign this submission on behalf of the Assignee.

In accordance with 37 C.F.R. § 3.73 Assignee hereby appoints as the attorneys of record and grants the sole power of attorney, with full power of substitution and revocation, for this application and for all transactions with the U.S. Patent and Trademark Office in connection therewith, to Richard E. Oney (Reg. No. 36,884) and Susan Stone Rosenfield (Reg. No. 36,287).

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U.S. Provisional Application No.: _____

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Respectfully submitted,

ARIZONA BOARD OF REGENTS,
a body corporate, acting for and on
behalf of ARIZONA STATE
UNIVERSITY

Dated: December 06, 2002

By: M. Ann Freudendahl
M. Ann Freudendahl
Interim Director of Licensing &
Intellectual Property Administration